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Hydralazine Modifies Aβ Fibril Formation and Prevents Modification by Lipids *in Vitro*†

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ABSTRACT: Lipid oxidative damage and amyloid β (Aβ) misfolding contribute to Alzheimer's disease (AD) pathology. Thus, the prevention of oxidative damage and Aβ misfolding are attractive targets for drug discovery. At present, no AD drugs approved by the Food and Drug Administration (FDA) prevent or halt disease progression. Hydralazine, a smooth muscle relaxant, is a potential drug candidate for AD drug therapy as it reduces Aβ production and prevents oxidative damage via its antioxidant hydrazide group. We evaluated the efficacy of hydralazine, and related hydrazides, in reducing (1) Aβ misfolding and (2) Aβ protein modification by the reactive lipid 4-hydroxy-2-nonenal (HNE) using transmission electron microscopy and Western blotting. While hydralazine did not prevent Aβ aggregation as measured using the protease protection assay, there were more oligomeric species observed by electron microscopy. Hydralazine prevented lipid modification of Aβ, and Aβ was used as a proxy for classes of proteins which either misfold or are modified by HNE. All of the other hydrazides prevented lipid modification of Aβ and also did not prevent Aβ aggregation. Surprisingly, a few of the compounds, carbazochrome and niclosamide, appeared to augment Aβ formation. Thus, hydrazides reduced lipid oxidative damage, and hydralazine additionally reduced Aβ misfolding. While hydralazine would require specific chemical modifications for use as an AD therapeutic itself (to improve blood brain barrier permeability, reduce vasoactive side effects, and optimization for amyloid inhibition), this study suggests its potential merit for further AD drug development.

Amyloid β protein (Aβ),[1] which misfolds and accumulates in Alzheimer's disease (AD) brains, is central to the "amyloid hypothesis" where Aβ causes AD pathology (*1*, *2*). This toxicity is in part due to increased oxidative damage (*3*−*8*) and the toxicity of oligomeric species of Aβ (*9*). Indeed, Aβ may play a direct role in this oxidative damage as it directly oxidizes many substrates, including lipids (*10*−*15*). Additionally, amyloid plaques, of which Aβ is the major component (*16*, *17*), contain transition metals (*18*−*24*) and are competent for generating oxidative stress (*18*, *25*, *26*). The oxidation products generated, such as H_2O_2 and reactive lipid oxidation products such as 4-hydroxy-2-nonenal (HNE), are likely mediators of toxicity in this disease.

Identification of compounds that can prevent these two pathological features of AD, oxidative damage and protein misfolding, could provide the basis for future drugs for AD. Hydralazine was selected as it is an excellent scavenger of reactive lipid oxidation products, such as acrolein and HNE, and also prevents the lipid modification and cross-linking of proteins (*27*−*35*). In addition to reducing reactive oxygen species and lipid peroxidation, hydralazine also prevented aldehyde-mediated cell death, NADPH/monoamine and xanthine oxidases, and NOS and COX-2 enzyme activities (*33*, *36*−*43*). The scavenging efficacy of hydralazine is primarily due to its nitrogen atoms in the ring and hydrazide group (*34*, *44*). Additionally, hydralazine effectively reduced Aβ production in primary neuronal cells from the brains of the Tg2576 AD transgenic mouse model (*45*). Thus, hydralazine's antioxidant function and reduction of Aβ production are attractive outcomes as a starting point for drug development for AD.

We evaluated the efficacy of hydralazine, and related hydrazides, to prevent (1) Aβ misfolding and (2) HNE protein modification using transmission electron microscopy and Western blotting methods. Two of the compounds exhibited pleiotropic effects on Aβ aggregation; hydralazine treatment appeared to increase Aβ oligomer formation while carbazochrome appeared to increase Aβ fibril formation. In general, all of the hydrazides tested, excluding nialamide, prevented lipid modification of Aβ. Thus hydralazine, following chemical modifications to improve blood brain barrier penetration and to reduce side effects, offers a potential starting point for AD drug development.

MATERIALS AND METHODS

Materials. HNE was obtained at a concentration of 10 mg/mL (Cayman Chemical, Ann Arbor, MI). Stock solutions of hydrazides (from Sigma-Aldrich and Thermo Fisher Scientific Inc.) were made to 100 mM. The compounds were dissolved as indicated: BSc3094 (Sigma), 2-hydrazino-4-phenylthiazole, indole-3-acetic hydrazide, phenelzine sulfate, 4-aminobenzhydrazide, and nialamide in DMSO; hydralazine, carbazochrome, and niclosamide in 0.10, 0.15, and 0.6 N NaOH in water; isonicotinic acid hydrazide and 1,1′-diphenylhydrazine hydrochloride in H_2O; and 1,1′-diphenylhydrazide hydrochloride in methanol.

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[1]Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; DMSO, dimethyl sulfoxide; HNE, 4-hydroxy-2-nonenal.

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All stock solutions were diluted into water prior to use. Lyophilized Aβ42 at >95% purity was purchased from rPeptide, Athens, GA, and stored desiccated at −80 °C, and a stock solution (0.5 mg/mL) was prepared by dissolving Aβ42 in hexafluoroisopropanol. For the experiments below, an aliquot of Aβ42 was lyophilized and then resuspended in 100 mM phosphate buffer (calcium and magnesium free) to a final concentration of 5, 11.5, or 15 μM as described below. The following antibodies were used for Western blotting: anti-HNE rabbit polyclonal HNE-11S (Alpha Diagnostic International, San Antonio, TX); anti-Aβ antibodies 2H4 (epitope 1−8), 6E10 (epitope 1−17), 4G8 (epitope 17−24), and 12F4 (C-terminal epitope of Aβ42) (Covance, Maryland Heights, MO); A11 (epitope to oligomeric Aβ) and OC (epitope to Aβ fibrils, both a gift from Rakez Kayed (*46*)), and NU4 (epitope to oligomeric Aβ, a gift from Dr. William Klein (*48*)), all used at dilutions of 1:2000. The goat secondary anti-mouse and rabbit HRP-linked antibodies (Invitrogen) were used at 1:2000 dilutions. The mass spectrometry grade proteases used for the protease protection assay were TPCK-treated trypsin reconstituted in 50 mM acetate (1 μg/μL; Pierce, catalog no. 90055), proteinase K (20 mg/mL at 30 units; NEB, catalog no. P8102S), and endoproteinase AspN (reconstituted to 20 mg/mL in water; Pierce, catalog no. 90053). All water used in experiments was purified to 18 mΩ using an ion exchanger and reverse osmosis (Purlab Ultra, Elga, Lowell, MA).

Amyloid Aggregation. Aβ42 was aggregated in 100 mM PBS, pH 7.4, at a concentration of 11.5 μM in 50 μL (in a PCR tube) and agitated in an Eppendorf thermomixer (37 °C, 14000 rpm; ThermoFisher Scientific, Pittsburgh, PA) for 24 h with and without hydrazides at concentrations of 25 and 100 μM. To test the protease protection and filter trap assays, Aβ was aggregated at 15 μM in 100 mM PBS, pH 7.4, at room temperature overnight.

HNE Modification of Aβ42. Aβ42 (5 μM) was modified by HNE (2.5 mM) via incubation at 37 °C overnight in 100 mM PBS, pH 7.4, with and without the hydrazides at concentrations of 25, 250, and 2500 μM. It is essential to use PBS free of magnesium and calcium ions, as these ions prevent HNE modification of Aβ. We also avoided the use of azide, primary amines, or ammonia in these methods as they quench the hydrazide reaction. The HNE reaction was quenched by addition of Tris and DTT contained within SDS sample buffer.

Western Blotting. For Western blotting analysis, Aβ and Aβ-HNE were electrophoresed on 16.5% Tris−Tricine PAGE gels and transferred onto 0.2 μm nitrocellulose membranes. The membranes were blocked with 5% fat-free milk, incubated overnight at 4 °C with antibodies to Aβ (6E10 monoclonal; Covance) or HNE (HNE 11S rabbit polyclonal; Alpha Diagnostic) used at 1:500 dilutions. Blots were then incubated with the secondary HRP detection antibody for 1 h at room temperature. All membranes were blotted with regular ECL reagents (Pierce) and then exposed to X-ray film (Pierce).

The conformation of Aβ with hydralazine was further probed using antibodies specific for either oligomeric (A11 and Nu4; gifts from Rakez Kayed (*47*) and Dr. William Klein (*48*)) or fibrillar Aβ (OC; a gift from Rakez Kayed (*47*)). For this experiment, 2 μL of the samples or ~260 ng (without SDS sample buffer) was spotted on PVDF membrane and allowed to adsorb to the membrane. The blot was dried in an incubator at 37 °C for 5 min, placed in 100% methanol and then water for 30 s each, and then blocked in 5% fat-free milk for 1 h. Thereafter, the sample was treated similarly to a Western blot.

Electron Microscopy. To analyze the morphology of oligomeric and fibrillar forms of Aβ, transmission electron microscopy (TEM) was used. Aliquots (1 μL) of Aβ with and without drug were pipetted onto carbon-filmed 200 mesh copper grids (EM Biosciences) and incubated in a humidified chamber for 5 min. Excess liquid was wicked away using filter paper. The grid was stained with 10 drops of filtered 2% phosphotungstate acid applied at a 45° angle. The samples were dried, and images of the negative stains were collected with an FEI Morgagni 268 transmission electron microscope at an accelerating voltage of 80 kV.

Filter Trap Assay. The filter trap method essentially allows unaggregated Aβ to filter through a membrane, while aggregated (Aβ or amyloid) is trapped in the filter (*49*−*51*). We utilized both nitrocellulose membrane (*51*) and a cellulose acetate membrane (OE66; Schleicher and Schuell). Aggregated or unaggregated Aβ (~340 ng) was equilibrated for 5 min in 500 μL of 2% SDS in water. The membrane was separately prequilibrated with 2% SDS for 5 min. The filter membrane was assembled into a slot blot apparatus (Bio-Rad), and the diluted Aβ sample in 2% SDS was filtered, followed by three washes of 200 μL of 2% SDS. Following filtration of the sample, the blot was carefully removed, washed in 2% SDS and then TTBS, blocked in 5% fat-free milk, and treated similarly to a Western blot.

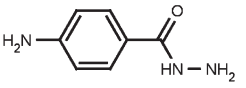
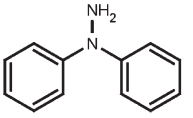
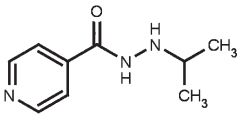
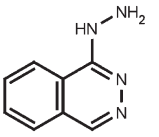
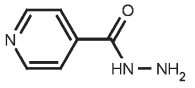
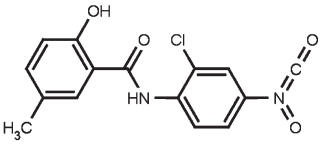
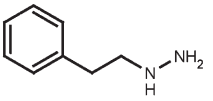
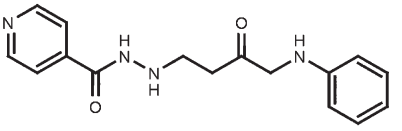
Protease Protection Assay. To further probe the conformation of Aβ, we developed a novel protease protection assay, modified from Giasson et al. (*52*), exploiting the knowledge that upon aggregation Aβ becomes protease resistant and antibody epitopes are protected (*53*). Aggregated or unaggregated Aβ (~260 ng) was digested with either proteinase K (0.66 μL of a diluted 1 mg/mL solution) in 100 mM PBS, trypsin (0.5 or 1 μL of the 1 μg/μL stock) in 50 mM ammonium bicarbonate, pH 8.45, or endoproteinase AspN (1 μL of the stock at 0.04 ug/μL) in 50 mM ammonium bicarbonate at pH 8.0. The final reaction volume for the proteolysis was 20 μL and carried out at 37 °C for ~14−18 h. The digested samples (26 ng) were dot blotted onto nitrocellulose, and the presence of protease-protected epitopes was probed using antibodies to Aβ (2H4, 6E10, 4G8, and 12F4 as described in Materials and Methods). Since the most effective reagents for differentiating between aggregated and unaggregated Aβ were trypsin and the 2H4 antibody, they were used for the protease protection assays.

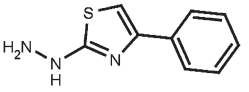
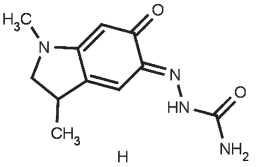
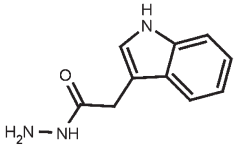
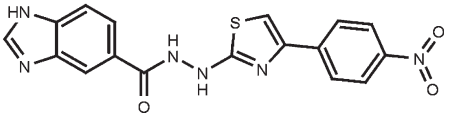
Cell Culture. PC12 cells were cultured in DMEM/F12 with 15% bovine serum in T25 flasks in a humidified chamber at 37% with 5% CO_2 and passaged 1/4 into new flasks every 3 days. For experiments, cells were plated on 12-well plates and used after allowing for overnight cell attachment.

Cell Treatment. Media from the cells were removed, and 200 μL of fresh media was added to the cells in the 12-well plate. This removed any nonadherent or dead cells prior to the experiment and allowed use of less reagent. Aβ (0.5 mg/mL) was lyophilized to remove the hexafluoroisopropanol and a working solution of 300 μM prepared in cell media and not DMSO, as DMSO concentrations of 10% resulted in cell death (data not shown). Hydralazine was dissolved at 100 mM in 0.1 N NaOH and then diluted to a working solution of 1 mM in cell culture media. Cells were either untreated or treated with Aβ42 (30 μM), hydralazine (100 μM (*29*, *39*)), or both Aβ and hydralazine for 24 h.

Measurement of Cell Death. Cell death was quantified using two separate methods using a cell counter or flow cytometry. Staining with trypan blue or propidium iodide was used to

Table 1: Chemical Structures of Compounds Tested[a]

Group A	SIngle Ring/ Spacing	Group B	Double ring/ Spacing
4-Aminobenzoyl hydrazide		1,1-Diphenylhydrazine	
Iproniazide		Hydralazine	
Isonicotinic hydrazide		Niclosamide	
Phenylzine		Nialamide	

Group C	Similar substructure
2-Hydrazine-phenylthiazole	
Carbazochrome	
Indole-3-3acetic acid hydrazide	
Phenylthiazole hydrazide BSc3094	

[a]The chemical compounds are grouped according to ring number/spacing and structural similarity.

differentiate between live and dead cells, where trypan blue is excluded from live cells with an intact cell membrane and propidium iodide is excluded from live cells and stains the nuclei of dead or dying cells. Cells were collected into 15 mL tubes and diluted to a final volume of 1 mL of calcium- and magnesium-free PBS (Invitrogen). For the trypan blue method, total cell numbers and trypan-stained cells were quantified using the Countess automated cell counter, as directed by the manufacturer (Invitrogen). The suspended cells (9 μL) were mixed with 1 μL of 0.4% trypan blue stain (Invitrogen) and analyzed in the Countess cell counting chamber slide which gave both cell numbers as well as cell viability. Cell death was also quantified by flow cytometry (Guava PCA-96 Base benchtop flow cytometer; Millipore) following the staining of dead cells using a standard propidium iodide protocol. Propidium iodide (10 μL of a 20 μg/mL solution) was added to 1 mL of suspended cells and analyzed in both the yellow and red spectrum using the Guava flow cytometer.

RESULTS

Structures of Compounds Tested. We evaluated the efficacy of several hydrazides to (1) inhibit Aβ misfolding and (2) prevent lipid modification by the reactive lipid HNE. The

structures of the compounds are shown in Table 1 and were selected based on similarity to existing anti-Aβ compounds, as well as grouped into single and double ring structures. For example, the following structures were similar to existing anti-amyloids: nialamide $\approx$ nordihydroguaiaretic (*54*), isonicotinic acid hydrazide and 4-amino benzhydrazide $\approx$ o-vanillin (*55*), 1,1′-diphenylhydrazine hydrochloride $\approx$ 4,4′-dihydroxybenzophenone (*54*), hydralazine $\approx$ napthoquinone and juglone (*55*), and finally indole-3-acetic hydrazide $\approx$ similar substructure to azure C (*55*) and myrecetin (*54*). Additionally, while hydrazides are reactive compounds, they have been and can be used as drugs. Examples of FDA-approved hydrazide drugs are hydralazine (Apresoline, antihypertensive), iproniazid phosphate (Euphozid, antidepressant), isonicotinic hydrazide (Laniazid, antituberculosis), nialamide (Niamid, antidepressant and anxiolytic), and niclosamide (Niclocide, teniacide).

Hydralazine Induces Aβ Oligomerization. Agitation of Aβ in the presence and absence of the drugs resulted in the formation of high molecular weight Aβ aggregates that did not penetrate the stacking gel. Aggregation of Aβ with 25 μM hydralazine or carbazocrome resulted in the formation of higher molecular weight species of Aβ (Figure 1A). At a higher concentration (100 μM), carbazochrome and hydralazine, as well as isonicotinic acid, resulted in high molecular weight oligomeric species (Figure 1B), suggesting that these compounds augmented Aβ aggregation. We used transmission electron microscopy of negatively stained Aβ fibrils, as an independent method to validate Aβ fibril formation.

Hydralazine and Aβ Fibril Formation. Aβ fibril formation was evaluated using transmission electron microscopy of negatively stained fibrils (100000× magnification, Figure 2). In the unaggregated Aβ, prepared from freshly lyophilized and reconstituted Aβ, some small oligomeric species were observed (Figure 2, Unagg); however, the aggregated Aβ had numerous negatively stained fibrils (Figure 2, Agg). These fibrils were short, which may have been due to breakage of longer fibrils upon agitation. Hydralazine was the only compound which reduced fibril formation, with small coiled structures (suggesting oligomers) and some fibrils being observed (Figure 2, hydralazine). Interestingly, the non-hydrazide compound, carbazochrome, which increased Aβ oligomers observed on the Western blots (Figure 1), appeared to enhance fibril formation (Figure 2, carbazochrome). The fibril staining appeared different for iproniazid, 2-hydrazine-4-phenylthiazole, and niclosamide; however, fibrils were evident (Figure 2, iproniazid, 2-hydrazine-4-phenylthiazole, niclosamide). Thus, there appears to be a pleiotropic effect on Aβ fibril formation, with carbazochrome enhancing and hydralazine reducing fibril formation.

The conformation of Aβ following hydralazine treatment was further analyzed using several methods: conformational specific antibodies, filter trap assay, and a novel protease protection assay. In this study the conformational specific antibodies did not differentiate between unaggregated and aggregated Aβ applied directly to nitrocellulose or PVDF membranes or following SDS−PAGE and Western blotting (data not shown). While the filter trap method effectively trapped only aggregated Aβ (data not shown), it did not appear specific when drugs were used. The concern was that, in the presence of drugs, Aβ was trapped nonspecifically within the membrane, especially that SDS precipitation of drugs could increase retention of Aβ in the filter/membrane. The last method tested was the protease protection assay (Figure 3A). Here the protease resistance of aggregated

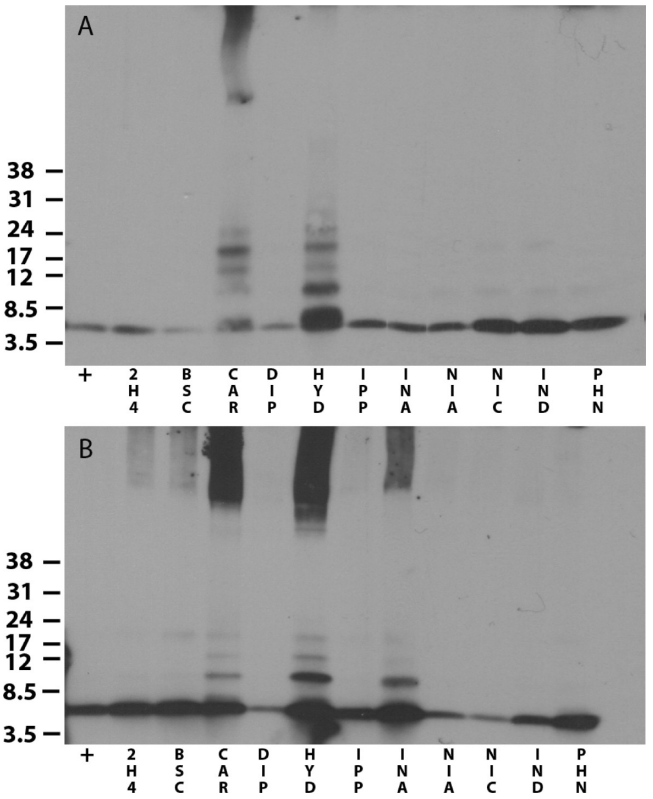


FIGURE 1: Hydralazine induces Aβ oligomerization. Aβ (11.5 μM) aggregated overnight alone or with (A) 25 μM or (B) 100 μM compound was electrophoresed on a 4−20% Tris−Tricine gradient gel and immunoblotted with the anti-Aβ antibody 6E10. Protein unable to enter the gel remains at the top of the gel, with monomer migrating at 4 kDa. Abbreviations: 2H4 = 2-hydrazine-4-phenylthiazole, BSC = BSC3094, CAR = carbazochrome, DIP = diphenylhydrazine, HYD = hydralazine, IPP = iproniazide phosphate, INA = isonicotinic acid hydrazide, NIA = nialamide, NIC = niclosamide, IND = indole-3-hydrazide, and PHN = phenylzine.

Aβ was used to advantage in order to immunologically differentiate between unaggregated (monomer Aβ) and aggregated Aβ (fibrillar Aβ). Proteolytic enzymes were selected (trypsin, proteinase K, and endoproteinase AspN) which all cleave specific Aβ antibody epitopes (Figure 3A). Presence of Aβ immunoreactivity indicates that Aβ is misfolded (fibrillar Aβ) and that the antibody epitopes were protected from proteolysis (Figure 3B). Several proteases were evaluated, and based on their cleavage sites in the primary sequence, trypsin and endoproteinase AspN will partially digest Aβ, while proteinase K will completely digest Aβ (Figure 3A). Proteinase K digestion resulted no immunoreactivity for any antibody, indicating complete digestion of all antibody epitopes regardless of Aβ conformation (Figure 3B). Shorter digestion times resulted in the ability to differentiate between aggregated and unaggregated Aβ (data not shown). Endoproteinase AspN digestion and the 2H4 antibody differentiated between aggregated and unaggregated Aβ (Figure 3B). Likewise, staining with the 2H4 antibody after trypsin digestion yielded immunoreactivity for aggregated but not unaggregated Aβ. The most effective antibody was 2H4, while 6E10 and 4G8 detected both forms of Aβ, and 12F4 was unreactive under these conditions (Figure 3B). Thus our data indicate that the trypsin cleavage site at Arg 5 in unaggregated, but not aggregated Aβ, disrupted the 2H4 antibody epitope for the 2H4 antibody. This protection from trypsin proteolysis in aggregated Aβ has been reported previously (*53*). Next, the protease protection assay (with the

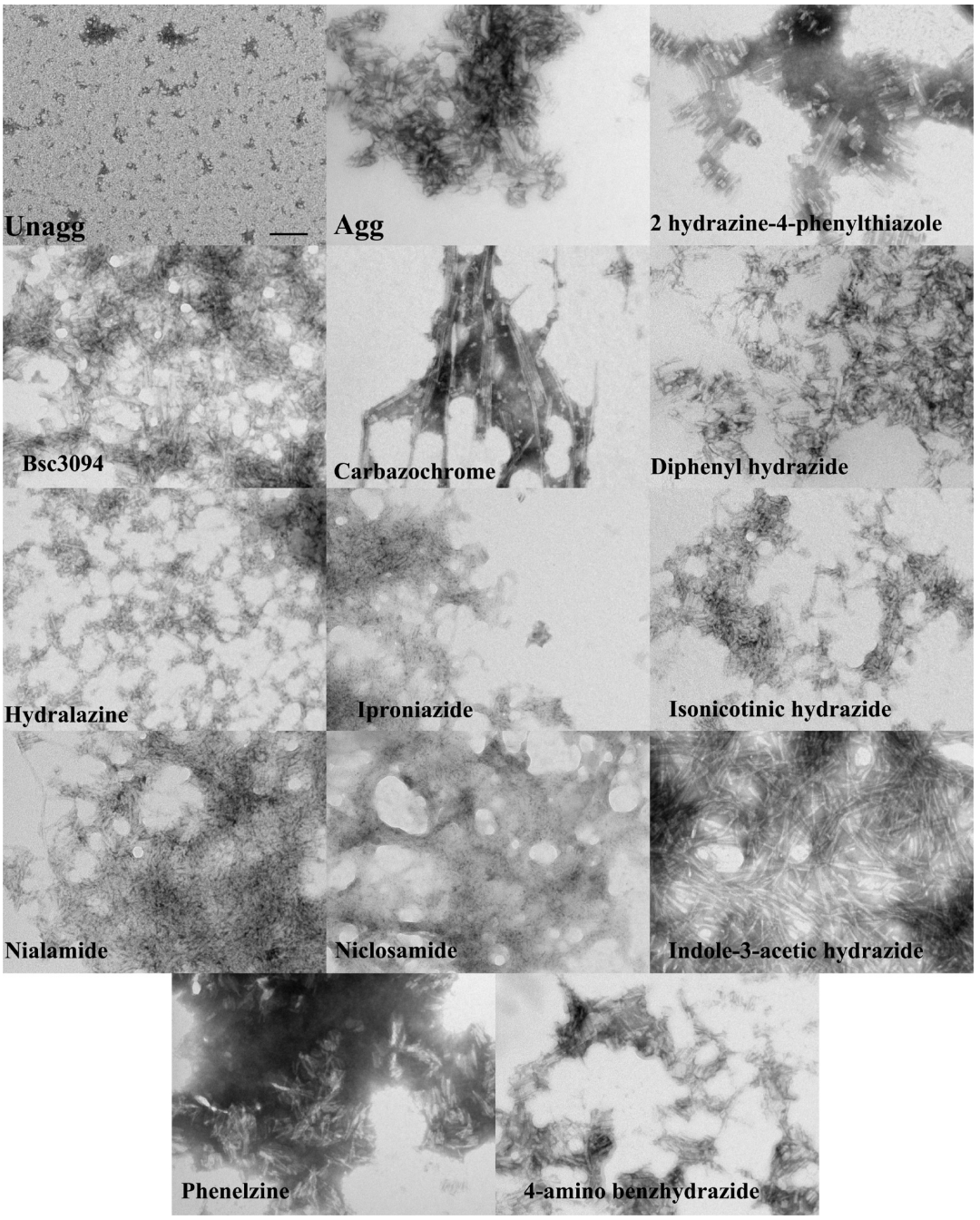


FIGURE 2: Hydralazine reduced Aβ fibril formation. Transmission electron microscopy of unaggregated (Unagg) or aggregated (Agg) Aβ (11.5 μM) with and without compounds and negatively stained with 2% phosphotungstate acid. Scale bar represents 100 nm.

trypsin digestion and detection with the 2H4 antibody) was used to analyze Aβ conformation following incubation with hydralazine (Figure 3C). Only aggregated (Pos) and not unaggregated Aβ (Neg) was detected using the protease protection assay (Figure 3C). Treatment of Aβ with 100 or 400 μM hydralazine yielded protease-resistant Aβ, indicating aggregation of Aβ (Figure 3C). Finally, higher magnification of Aβ treated with hydralazine (180000$\times$ magnification of the same field as in Figure 2, hydralazine at 100000$\times$ magnification) indicated the presence of short coiling Aβ species (suggesting oligomers) and some short fibrils (Figure 3D). Treatment of cells with hydralazine, Aβ, or both hydralazine and Aβ did not cause significant cell death as compared to the untreated controls (4%; data not shown). The fact that Aβ did not cause cell death suggested that this may be due to conformation, with Aβ at 30 μM only forming fibrils. Aβ used at 10 μM also did not result in cell death (data not shown). A separate set of experiments as above performed previously by another researcher also did not result in death of PC12 cells or primary mouse neuronal cells (data not shown, Dr. Gregory Bix).

Hydrazides Prevent Lipid Modification of Aβ. We then determined whether hydrazides could prevent the oxidative modification of Aβ by HNE, a reactive product of lipid oxidation, using Western blotting. Aβ electrophoresed as a ~4 kDa band (Figure 4, Aβ −,+), and modification of Aβ (5 μM) with an excess of HNE (2.5 mM) resulted in a shift in the electrophoretic mobility of Aβ to higher molecular weight structures as detected by an anti-Aβ antibody (6E10). This modification generally resulted in a reduction in the levels of monomeric Aβ (Figure 4, Aβ). Hydrazide drugs (25, 250, and 2500 μM) coincubated with

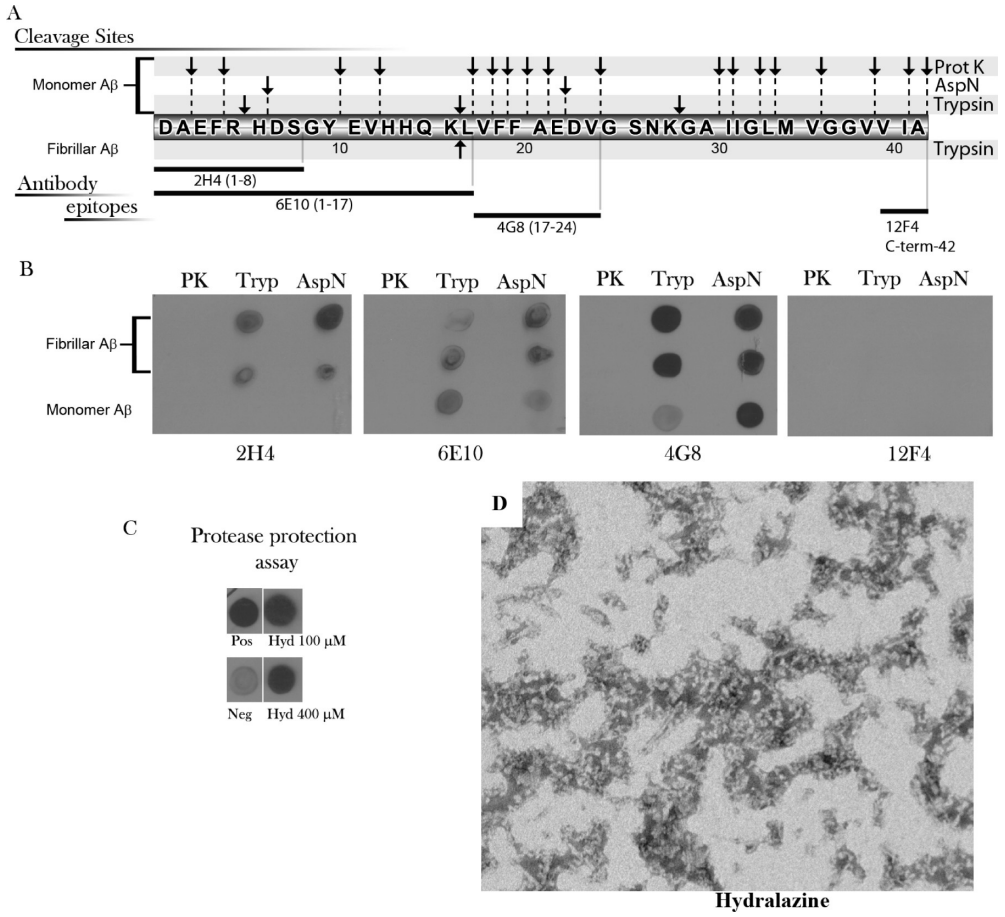


FIGURE 3: Effect of hydralazine on Aβ misfolding. (A) Diagram of the protease protection assay depicting protease cleavage and antibody epitope sites within Aβ42. Here the protease resistance of aggregated Aβ was used to advantage in order to immunologically differentiate between unaggregated (monomer Aβ) and aggregated Aβ (fibrillar Aβ). Protease cleavage sites (arrows) are shown for proteinase K (Prot K), endoproteinase AspN (AspN), and trypsin (trypsin). Cleavage within unaggregated Aβ (monomeric Aβ) and aggregated Aβ (fibrillar Aβ) are shown above and below the Aβ primary sequence. These sites within monomeric Aβ were determined using peptide cutter (http://ca.expasy.org/tools/peptidecutter/). The cleavage sites for trypsin within fibrillar Aβ are shown below the Aβ primary sequence (*53*), while the sites for proteinase K and AspN are unknown and thus not depicted. The epitopes for the antibodies to Aβ are depicted as horizontal lines. (B) Evaluation of protease enzymes and antibody combinations for the protease protection assay for aggregated (fibrillar Aβ) and unaggregated (monomer Aβ) Aβ. Aβ was aggregated overnight either at 11.5 μM shaking at 14000 rpm in an Eppendorf thermomixer at 37 °C (top row) or at 15 μM at room temperature (middle row). This allows for application of the protease protection assay to different Aβ aggregation protocols. The enzymes used were proteinase K (PK), trypsin (Tryp), and endoproteinase AspN (AspN). Following an overnight digestion of Aβ with enzymes, ~26 ng of Aβ was spotted onto a nitrocellulose membrane and detected with the indicated Aβ antibodies. (C) The best combination for the protease protection assay were trypsin digestion and 2H4 antibody detection. Application of the protease protection assay was applied to a dot blot of aggregated Aβ (Pos), unaggregated Aβ (Neg), and Aβ treated with 100 and 400 μM hydralazine (Hyd). (D) The transmission electron microscopy of Aβ aggregated with hydralazine, a higher magnification (180000×) of the same electron microscopy grid used in Figure 2.

Aβ and HNE resulted in a dose-dependent shift in the molecular weight to that of the monomeric form, with greatest efficacy at equimolar concentrations (2.5 mM HNE and drug, Figure 4, Aβ). Notable exceptions were 2-hydrazine-4-phenylthizole, hydralazine, and iproniazid, which had marked increase in intensity of higher molecular weight bands (Figure 4, Aβ: 2H4, HYD, IPP, and 4AH) than observed for Aβ without the drug. For these compounds, there was also an increase in the monomer band, indicating a reduction in HNE-adduct formation. This prevention of HNE modification was not altered by secondary structure and side group attached to the hydrazide, with the exception of nialamide (Figure 4, Aβ: NIA). To confirm a lack of HNE-adduct formation, immunostaining was performed with antibodies to HNE (Figure 4, HNE). The HNE-adduct blot mainly demonstrated the presence and absence of HNE adducts simply as a monomeric band, where all of the hydrazides, with the exception of nialamide, prevented HNE modification of Aβ (Figure 4, HNE). Carbazochrome, a non-hydrazide, did not prevent HNE modification (Figure 4, HNE). Together, these data indicate that the hydrazide moiety in the compounds prevents HNE modification of Aβ, and with the exception of nialamide, side groups attached to the hydrazide do not appear to block scavenging activity.

DISCUSSION

The major findings of this study are that (1) hydralazine alters Aβ fibril formation, likely with increased oligomeric species, and (2) hydrazides prevent lipid-mediated oxidative damage of Aβ. We have thus identified that hydralazine can serve as a template compound for further optimization for anti-Aβ drug development for AD.

Oxidative damage is a major pathological feature of AD, and preventing this damage may reduce related pathogenesis. Hydrazides have antioxidant activity and react with aldehydes and ketones (including pyruvic acid and α-ketoglutaric acid) to form hydrazones (*44*). Additionally, hydrazides also react with acids,

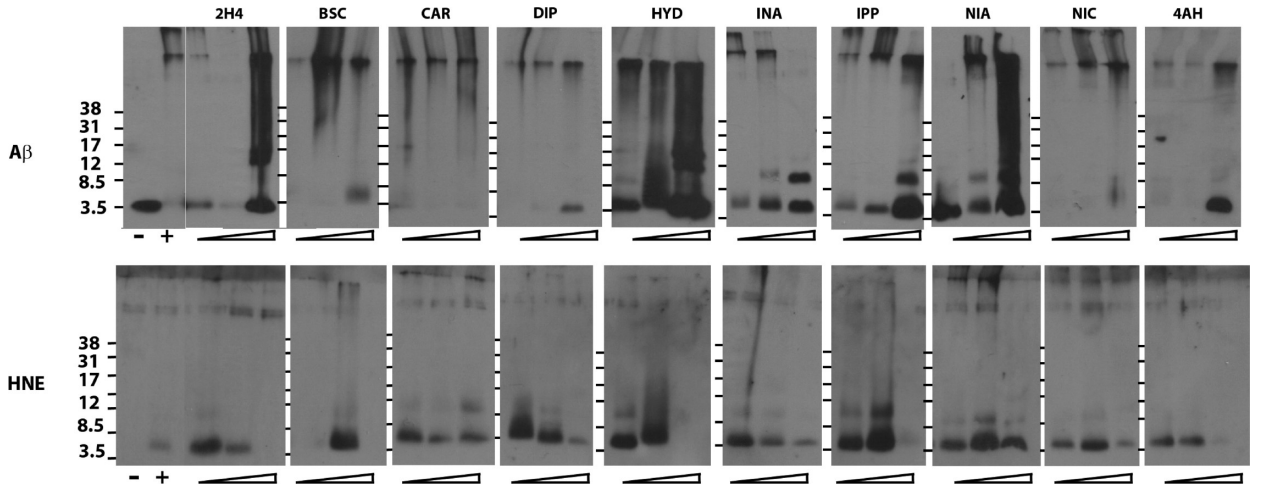


FIGURE 4: Hydrazides prevent lipid modification of Aβ. Aβ (5 μM) incubated with and without 4-hydroxy-2-nonenal (HNE, 2.5 mM) overnight at 37 °C was electrophoresed on a 4−20% Tris−Tricine gradient gel and immunoblotted with the anti-Aβ antibody 6E10 (Aβ) or HNE 11S anti-HNE-adduct antibody (HNE). The compounds, at 25, 250, and 2500 μM, were coincubated with Aβ incubated with HNE, with the increasing drug concentration represented by the triangle. Abbreviations: 2H4 = 2-hydrazine-4-phenylthiazole, BSC = BSC3094, CAR = carbazochrome, DIP = diphenylhydrazine, HYD = hydralazine, IPP = iproniazide phosphate, INA = isonicotinic acid hydrazide, NIA = nialamide, NIC = niclosamide, IND = indole-3-hydrazide, PHN = phenylzine, and 4AH = 4-aminobenzhydrazide.

acid chlorides, esters, anhydrides, and azides (*44*). This reactivity contributes to hydralazine's prevention of lipid aldehyde modification of proteins and Aβ (*27*−*35*), which we demonstrated to be relatively independent of structure and the side group attached to the hydrazide (with the exception of nialamide). In addition to preventing carbonyl modification of proteins, hydralazine also reduced reactive oxygen species, lipid peroxidation, and aldehyde-mediated cell death and, furthermore, prevented NADH/monoamine, xanthine oxidases, NOS and COX-2 enzyme activities (*33*, *36*−*43*). Although the antioxidant effect of hydralazine is likely only effective extracellularly and not intracellularly (ref *34* and personal communication from Dr. Burcham), this drug can still be potentially used for extracellular amyloid plaque therapy in AD. Thus, hydralazine prevents oxidative damage *in vitro*.

The reported protective *in vivo* effects of hydrazides are discordant, which may be due to both the dosage and the generation of free radicals by hydrazides (*56*−*60*). Protective effects of hydralazine include reduced oxidative damage to proteins during hypertension, atherosclerosis, and spinal cord injury (*35*, *42*, *61*, *62*). However, other reports have indicated that hydrazides do not reduce oxidative damage (*63*, *64*). The latter studies, with no efficacy of hydralazine, used higher doses of hydralazine than studies showing benefit (20 and 50 mg/kg per day vs the effective studies at 5 and 15 mg/kg per day). Thus, this may be a concentration effect, as hydrazides decompose in aqueous solution, catalyzed by trace amounts of transition metals, to form radicals on the hydrazide group and generate $O_2^{\bullet -}$ and H_2O (*60*). These radicals result in concomitant damage to DNA and degradation of proteins. Hence, lower doses of hydralazine, similar to those used in humans (3 mg/kg per day for a 60 kg human), may be protective. Finally, modification of hydralazine to improve blood brain barrier permeability will allow for lower concentrations to be used, to reduce concentration-dependent side effects, and to improve effects on cognition. Thus, hydralazine is a potential drug to reduce oxidative damage in AD pathology.

One of the other pathological features of Alzheimer's disease is protein misfolding, and we focused on Aβ misfolding. Aβ misfolding is a multistep process and can be inhibited at several points along the pathway. Aβ misfolding is a nucleation-dependent process resulting in conversion of the monomer, after a lag time, to sequentially form oligomers, protofibrils, and then fibrils (*65*). The central hydrophobic cluster 17−21 region KLVFF (*66*) seeds misfolding, with β-sheet formation at residues 18−26 and 31−42 (*67*). The seed or oligomer is likely a β-sheet structure with intraprotein interactions, with the C-shaped conformation having a salt bridge between K28 and D23 and internal contacts at residues L17/F19 and I32, L34, V36 (*68*). This structure facilitates the formation of protofibrils, likely via steric zippering of the quaternary structure via M35 and G33/37 residues (*68*, *69*). Thereafter, the protofibrils associate laterally to form fibrils (*53*, *65*, *70*). This lateral association likely involves the amino-terminal region (*71*). Thus, Aβ misfolding can be halted at the monomer, oligomer, or protofibril stages.

Common features of Aβ inhibitors include specific ring structure (planar with motility) and aromatic interaction with aromatic sequences in the amyloidogenic core of Aβ (*72*−*77*). These properties enable the molecule to interact with Aβ via π−π ring interactions as well as intercalate between Aβ fibrils. Hydralazine possesses these features, and additionally its ring structure is similar to the anti-Aβ compounds naphthoquinone, juglone (*55*), and clioquinol/PBT2 (*78*−*81*). While we initially thought that hydralazine inhibited Aβ aggregation at the oligomer stage, an additional method (protease protection assay) did not support this finding. This finding is similar to a previous study (*45*) where hydralazine was unable to prevent oligomerization. However, oligomeric Aβ may also exhibit protease protection. Differentiation between oligomeric and fibrillar Aβ may be attained with other proteases which do not cleave at the amino terminal (which could be sequestered in Aβ oligomers). For example, chymotrypsin is such an enzyme as it cleaves in the center of the Aβ sequence (Phe 20) and within the 4G8 antibody epitope (17−24) (*53*, *71*). Another mechanism by which hydralazine may interact with Aβ is via metal binding. Hydralazine binding to metals, a known property of this compound (*44*, *82*, *83*), may augment hydralazine interaction with metals bound to the amino-terminal region of Aβ. Thus, hydralazine may sequester

Aβ in the oligomeric stage, and additional assays are required to rule out fibril formation.

The question remains whether hydralazine can be used as an anti-Aβ drug. In short, it can be used as a proof-of-principle compound to evaluate effects on reduction of Aβ production (thus prevention of fibril formation) and antioxidant activity in an animal model of AD. Interestingly, hydralazine has not been tested for cognitive benefits in a *transgenic* model of AD, with one research group demonstrating no effect on cognition with intracerebral Aβ injections (*84*). We anticipate that the activity of hydralazine on reactive oxygen species, lipid peroxidation, aldehyde-mediated cell death, NADPH/monoamine and xanthine oxidases and NOS and COX-2 enzymes, in combination with reduction in Aβ production (*45*) (likely via reduction of oxidative stress), will reduce pathology in a transgenic model of AD. Additionally, hydralazine functions as a metal chelator (*44*, *82*, *83*), and metal chelation reduces both plaque load (*78*, *79*, *81*) and transition metal catalyzed oxidative damage. Thus, hydralazine is a good scaffold compound for an anti-AD compound, with potential to reduce Aβ production, Aβ misfolding, and oxidative damage in AD.

The use of hydralazine in AD, however, has some drawbacks due to its (1) vasoactive effects, (2) free radical generation, and (3) reduced blood-brain barrier permeability. Chemical modification of hydralazine may overcome these drawbacks and retain beneficial effects, as discussed below. The structure C═N−N═C−NH−NH_2 represents the vasoactive pharmacophore (see hydralazine structure in Table 1), and replacement of the hydrazide group also reduces vasoactivity (*44*). Interestingly, the hydralazine scavenging activity may be separated from the vasoactive pharmacophore as this activity only requires the hydrazide group, and unlike vasoactivity, it is less sensitive to the location of nitrogen atoms in the ring (*34*). Finally, the antihypertensive effect can result in tachyphylaxis, which can be reduced by coadministration of β-blockers and a diuretic (*85*). Several hydralazine analogues are commercially available (*34*) and can be used to test such modifications. Thus, modification of hydralazine, retaining the antioxidant moiety and increasing the hydrophobicity, will likely increase blood-brain barrier permeability and its efficacy as a potential anti-AD drug.

Thus, hydralazine can serve as a scaffold molecule, with chemical modification to improve blood-brain barrier penetration and to reduce vasoactive effects, to prevent lipid oxidative damage as well as the modification and misfolding of Aβ in AD. However, it serves as a proof-of-concept drug for future development of potential therapies for AD.

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